Identification, Isolation and Genomic Characterization of Porcine Astrovirus in Shandong Province, China in 2021–2023

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Abstract **[Objective]** The paper was to identify, isolate, and characterize porcine astrovirus in Shandong Province between 2021 and 2023. **[Method]** A total of 1 025 samples of porcine diarrhea samples were collected from various regions of Shandong Province between January 2021 and October 2023. The samples were tested by RT–PCR, followed by sequencing and phylogenetic analyses of the polymerase. **[Result]** The total positive rate of PAstV was 34.6% (355/1025). The respective proportions of individuals infected with PAstV-1, PAstV-2, PAstV-4 and PAstV-5 were 25.4% (90/355), 28.2% (100/355), 35.2% (125/355) and 22.5% (80/355), respectively. Additionally, mixed infection was observed. Meanwhile, 849 samples of healthy pigs were tested by RT-PCR, and the results demonstrated that the total positive rate of PAstV was 8.13% (69/849). Of these, the proportion of PAstV-1, PAstV-2 and PAstV-4 infection was 27.5% (19/69), 37.7% (26/69) and 40.6% (28/69), and a mixed infection also existed. Further sequencing and characterization of some the selected isolates revealed low sequence identities (56.2%) with known PAstV strains, indicating the presence of novel types or genotypes of PAstVs. Furthermore, the isolation conditions of porcine astrovirus were optimized, resulting in the purification of a pure PAstV-4 strain (designated PAstV-4-GRF1). The virus was found to exhibit typical astroviral morphology, with nucleotide identity ranging from 89.9 to 95.4% with previously published PAstV-4 strains. Then, macrovirus transcriptome sequencing showed that 88.30% of the GRF1 samples were mammalian astroviruses. By species classification, PAstV 4 and PAstV 2 accounted for 21.79% and 0.32%, respectively. Phylogenetic tree analysis showed that the c15050 fragment was identical to the GRF-1 sequencing fragment of the isolated strain, and exhibited the highest homology with the Hunan PAstV-4 sequence MK460231 in China. **[Conclusion]** As the inaugural isolated PAstV-4 strain, it furnishes pivotal material for the investigation of the biological and pathogenic properties of this virus as well as for the prospective development of relevant biological and diagnostic reagents.

Keywords Porcine astrovirus; Epidemic situation; Genotype analysis; Isolation and identification

The astrovirus (AstV) is a member of the Astroviridae family (Astroviridae), which also includes mamastrovirus and avastrovirus. It is widely distributed in humans, mammals and birds, and can cause diarrhea and neurological symptoms[1–2]. The virions are about 28 –30 nm in diameter, non-enveloped, and contain a single-stranded, positive sense RNA molecule of 6.4 to 7.7 kb[3]. The genome is arranged in a 5' end that encodes non-structural proteins, three open reading frames (ORF1a, ORF1b, and ORF2), and a 3' end that encodes structural proteins[4]. As RNA viruses, they could facilitate the emergence of new viruses through gene reassortment or mutation, which could result in the transmission of genotypes across species[5–7].

Porcine astrovirus (PAstV) was first identified in 1980 by Bridger[8], and subsequently disseminated to China[9], the United States[10], Japan[11], South Korea[12], India[13], Chile[14] and other countries. At present, five genotypes of PAstV (PAstV-1 to PAstV-5) have been identified worldwide, with varying prevalence rates[1,15]. With the exception of PAstV-3, which is associated with neurological symptoms, all four types are associated with diarrhea[16–18]. Their pathogenicity is not well understood due to the difficulty in isolating them *in vitro* for experimental infections. To date, PAstV-1 was the first PAstV that has been isolated in cell culture[19]. Experimental infections with isolated strains of this virus have confirmed its pathogenicity, presenting as

Received: 2024–03–11 Accepted: 2024–04–23
Supported by Sub-project of the National Key Research and Development Program(2023YFD1800804-06); National Natural Science Funds of China (32302918 and 32302919); Innovation Capacity Improvement Project for Technology-based SMEs in Shandong Province (2023TSGC0006); Science and Technology Cooperation Project of Shandong and Chongqing (2022LYXZ030); Shandong Province Key R&D Program Rural Revitalization Project (2023TZXD083).
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mild diarrhea in piglets with damage to the villi of the small intestinal mucosa and eventual growth retardation[20]. In 2020, a swine astrovirus type 5 was isolated from clinical swine fever virus (CSFV)-infected tissue samples. This isolation may have been influenced by the fact that CSFV counter infection significantly enhanced the replication of PAstV-5[21]. However, it has been reported that the most prevalent strain reported in China was PAstV-4, while PAstV-2 and PAstV-4 were the most common throughout Asia [22]. The genetic characterization of PAstV in China remains relatively understudied. Consequently, there is a pressing need to identify, isolate, and assess the genetic diversity of PAstV currently present in Shandong, China.

1 Methods

1.1 Sample collection, cDNA synthesis and PAstV detection To explore the prevalence of PAstV strains in Shandong Province of China, a total of 1 025 diarrhea cases and 849 healthy pig samples from swine were collected from pig farms in 11 breeding regions, including Binzhou, Dezhou, Dongying, Heze, Jinan, Jining, Liaocheng, Tai'an, Weifang, Yantai and Zibo, from January 2021 to October 2023.

The RNA from the aforementioned samples was purified from the supernatant of a previously homogenized 10% suspension in PBS with RNAiso Plus (Takara, Japan) according to the manufacturer's instructions. The cDNA was reverse-transcribed in accordance with the instructions provided by the PrimeScript™ RT Reagent Kit (Accurate Biology, China) and subsequently used in a PCR amplification, employing specific primers for the detection of PAstV. Furthermore, positive samples were analyzed and genotyped via PCR using specific primers. The sequences of the primers utilized in this study are presented in Tab.1.

1.2 Sequence alignment and genotype analysis The PAstV-positive samples from the infected farms were further subjected to genome sequencing. To obtain the sequences, PCR was conducted using 2× Accurate *Taq* Master Mix (dye plus) (Accurate Biology, China) to amplify the overlapping fragments, using the primer pairs listed with the above. The reaction system was as follows: 94 ℃ for 30 sec; 35cycles of 98 ℃ for 10 sec, 56 ℃ for 30 sec and 72 ℃ for 1 min; final extension at 72 ℃ for 2 min. The amplified products were sent to Sangon Bioengineering (Shanghai) Co., Ltd. for sequencing. The obtained sequences were then used to compare with the reference strains registered in GenBank (Tab.1). Subsequently, phylogenetic trees were constructed using MEGA 6.0, employing the neighbor-joining method and the p-distance model to analyze the relationship between the PAstV gene and the reference strains.

Tab.1 Primers and PCR reaction conditions

Primers	Primer sequences (5′→3′)	Annealing temperature//℃	Product size//bp
PAstV-H-F	GACATTTTGTGGATTTACAGTTGG	51	279
PAstV-H-R	TTGGTCCTCCCCTCCAAAG	56	
P1-F	TCCTGTGCTATCAGTTGCTCTC	61	419
P1-R	GATTGCTGGTTTTGGACCTGTG	61	
P2-F	AGCAGCTGGATCGTCTTTGGA	59	826
P2-R	AGATTCAGCATCCCAGGTTGTT	59	
P4-F	TGGCTTCAGGCCTTTGAGTTTT	59	558
P4-R	CACCGTCGTAGTAGTCGTGAC	61	
P5-F	TGGTACGTRCACAATCTGTTGAA	57	181
P5-R	TCAGTGTCTTCCCAACCRTC	57	
PEDV-F	TTGAACCTAACACACCTCCT	56	324
PEDV-R	TAAGCTTGTCAGGGTTTTCG	56	
RV-F	TGTACTAGCACCATTCGTCA	56	616
RV-R	TCTTATTGTGCATGTAGCGG	56	

1.3 Cells and virus The PK-15 cell line (pig kidney epithelial cells, ATCC CCL-33) proved to be an optimal choice for the isolation and culture of PAstV *in vitro*. The samples were maintained in 10% FBS DMEM medium at 37 ℃ under 5% CO_2. The supernatant of the samples with positive PAstV was collected, filtered and sterilized through a 0.22 μm sterile filter, and then inoculated with PK-15 cells. Briefly, the prepared PK-15 cells (6×10^6 cells/mL) were treated with 500 μL of the sterilized sample supernatant and 500 μL of pre-configured TPCK trypsin solution (TPB 150 μL, trypsin 250 μL, DMEM 50 mL). After the incubation of 12h, the virus solution was discarded and replaced with 2% FBS DMEM, and then cultured in a 5% CO_2 incubator at 37 ℃ for 48 h. Total RNA was isolated from virus-infected cells that had been frozen and thawed three times, and the presence of PAstV was detected by RT-PCR.

PAstV type 4 strain, designated as PAstV4 GRF-1 was isolated and obtained from the diseased pigs in Jinan, Shandong Province, China. The virus was continuously passaged on PK-15 cells for over 18 generations, with a virus titer reaching $10^{4.5}$ TCID50/0.1 mL.

1.4 Scanning electron microscopy Briefly, the samples were fixed in 1% paraformaldehyde and 0.25% glutaraldehyde, dehydrated by an ascending alcohol series, and dried at the critical point with a Balzers CPD 030 Critical Point Dryer (BAL-TEC, Schalksmühle, Germany). Following the application of a gold/carbon coating to the samples using a sputter coater SCD 050, scanning electron micrographs were obtained using a LEO 1525 (Zeiss, Oberkochen, Germany).

1.5 Metaviral transcriptome sequencing analysis Total RNA was extracted from the fecal supernatant using TRIzol, and ribosomal RNA was removed from the total RNA of the extracted sample to obtain mRNA. Subsequently, the obtained mRNA was randomly fragmented into 250–300 bp short fragments with divalent cations in NEB Fragmentation Buffer. The fragmented RNA was employed as the template and the random oligonucleotide served as the primer for the synthesis of the first strand of cDNA. Afterwards, DNA libraries were constructed using the NEBNext® UltraTM DNA Library Prep Kit for Illumina (E7370L, New England Biolabs, Frankfurt am Main, Germany). The obtained raw data underwent sequencing quality analysis, during which adapters and low-quality reads were removed. This was conducted using FastQC for sequencing quality analysis. Kraken software was employed to classify and annotate the quality-controlled raw data, while SPAdes software was used for de novo assembly, thus obtaining the genome sequence. The spliced sequences were subjected to a comparison with the GenBank Virus RefSeq protein database, using the DIAMOND software, with the objective of identifying viral sequences and aligning the sequence predicted by ORF with the virus sequence extracted from the NR database of NCBI. The predicted PAstV sequence and the reference sequences of different genotypes of porcine astrovirus in GenBank were used to construct an evolutionary tree in MEGA7.0, employing the Neighbor-Joining method to construct the phylogenetic tree.

2 Results and Analysis

2.1 RT-PCR detection of PAstV A total of 1 025 diarrhoeic pigs and 849 healthy pigs from 11 pig farms in Shandong Province, China, were subjected to RT-PCR detection of PAstV. The overall prevalence of PAstV specific target genes in diarrhoeic pig samples was found to be 34.63% (355/1025), as evidenced by the expected size of the target band at 279 bp (Fig.1A). The total incidence rate of healthy pigs was 8.13% (69/849), indicating that PAstV is widely distributed in Shandong Province and an important cause of piglet diarrhea. Additionally, the co-infection with other diarrhea viruses, including PEDV and PRV, was analyzed. As a result, the rate of PEDV and PRV co-infection in diarrheal samples from pigs positive for PastV was 46.5% (165/355) and 42.3% (150/355), respectively (Fig. 1B). The co-infection rate of PEDV and PRV in PAstV-positive samples from normal pigs was 47.8% (33/69) and 43.5% (30/69), respectively (Fig.1C), indicating that they may play a potential role in PAstV-induced diarrhea in piglets.

In order to ascertain the genotype of the positive samples identified above, a RT-PCR was conducted using the specific primers. As shown in Fig.1D, the proportion of PAstVs in healthy pig samples was as follows: 27.5% (19/69) for PAstV-1, 37.7% (26/69) for PAstV-2, and 40.6% (28/69) for PAstV-4. A total of four groups of PAstVs were identified in samples of pig diarrhea samples: PAstV-1, PAstV-2, PAstV-4 and PAstV-5. The proportion of PAstVs was as follows: 25.4% (90/355) for PAstV-1, 28.2% (100/355) for PAstV-2, 35.2% (125/355) for PAstV-4, and 22.5% (80/355) for PAstV-5 (Fig.1E), with PAstV-4 accounting for the highest ratio. This indicates indicating that PAstV-4 was the predominant virus in diarrheic piglets in Shandong province.

2.2 Phylogenetic analysis of PAstV In the current study, a total of 29 strains were successfully sequenced from the 424 PAstV-positive samples. Here, a phylogenetic tree was constructed based on the ORF genes of the 29 identified PAstV strains and 75 reference AstV strains.

In order to ascertain the degree of homology between the eight sequenced fragments of PAstV-1 and the PAstV type 1 sequences in GenBank, a comparison was conducted. The results showed that the homology between the eight strains in Shandong was 73.4%–95.2%, with the homology with the representative strains AB037272, HM756258, GQ914773, and KF787112 being 77.2%–94.4%. One of the eight strains (811-3-1) in the branch of PAstV-1 showed a higher genetic relationship with strain KF787112 from China in 2013, sharing 94.4% of mean nt/aa identities. Phylogenetic trees were constructed based on the nucleotide sequences obtained in this study, which were compared with those of selected AstV sequences from other species available in GenBank. It is anticipated that all other identified PAstV-1 strains (circles in red) in conjunction with the five reference strain sequences will constitute the PAstV-1 clade, which is the most closely related to KF787112, with an average genetic distance of 0.137. Moreover, phylogenetic analysis revealed a close relationship between PAstV-1 and other astroviruses recovered from cats and tigers (Fig.2A).

For PAstV-2, the current 7 sequences showed identities of 60.8%–94.7% between each other, with 52.4%–86.2% identity to other available PAstV-2 sequences in GenBank, indicating significant variation of PAstV-2 in Shandong Province. Phylogenetic analysis indicated that the current PAstV-2 sequences from Shandong province clustered into a monophyletic group. For example, the isolated strains 625-7-2 and 719-5-2 are closely related to the American strain JF713712 in 2010 and the Japanese strain LC201586 in 2015. The strains 719-6-2, 1112-19-2, 1112-22-2 and 1112-24-2 were found to cluster together with the Ugandan strain KY940077 in 2012. Interestingly, AstV sequences recovered from roe deer (MN150125) and cow (HQ916313) also clustered with the PAstV-2 group, which may indicate a recent cross-species transmission (Fig.2B).

The 13 PAstV-4 sequences exhibited

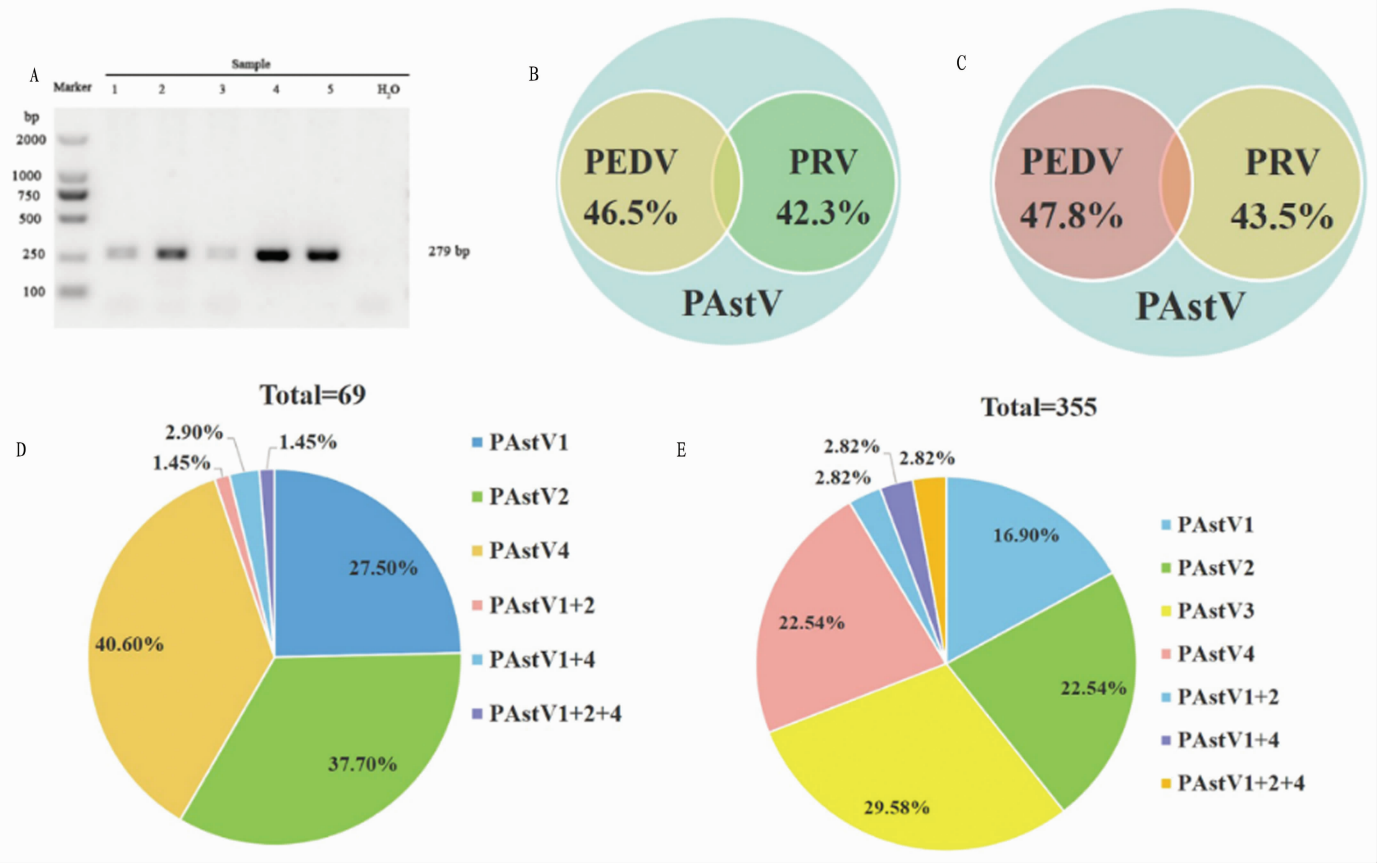

Fig.1 Prevalence of PAstVs in piglets

Note: A. Detection of astrovirus in diarrhea samples by RT-PCR (intend band 279 bp); B. The co-infection of PAstV with other diarrhea viruses in diarrheic piglets; C. The co-infection of PAstV with other diarrhea viruses in healthy piglets; D. The infection rate of the 3 porcine pathogens in the 69 PAstV-positive samples; E. The infection rate of the 4 porcine pathogens in the 355 PAstV-positive samples.

a degree of sequence identity ranging from 56.2% to 95.5%. Among them, the three isolated strains (1015-21-4, 1015-23-4 and 1015-24-4) exhibited relatively large variation with the other 10 strains, and their homology with the reference strains was also very low (60.5% –71%). The phylogenetic analysis revealed that the present sequences exhibited considerable divergence from those of various reference strains of PAstV-4, which were distributed globally. It is noteworthy that 1015-1-4 and 1015-4-4 exhibited a high degree of similarity to the GenBank wild boar astrovirus sequence (KX033447) from Jiangxi province in China, with genetic distances of 0.056 and 0.139, respectively (Fig.2C). A single strain of PAstV-5 was isolated and subjected to sequencing. The resulting homology between this strain and the reference strain was found to be between 83.8% and 86.5%. The phylogenetic tree showed that the strain was closely related to the 2017 Chilean strains MZ819170, MZ819171, and MZ819172 (Fig.2D).

2.3 Isolation and identification of PAstV-4 To obtain a pure PAstV isolate from diarrhea viruses, a total of 29 PAstV-positive diarrhea samples were selected and treated as a virus stock solution for the purpose of isolating the virus on PK-15 cells. However, neither RT-PCR nor cytopathic changes could detect PAstV after passages 3, except for PAstV-4 (named GRF-1). Following the infection of the cells by the GRF-1 sample, the cells exhibited a notable increase in size and roundness. Over the course of 24 h, the cells gradually disintegrated and fell off. Following the freezing and thawing of the cell liquid on three occasions, the subsequent passage was initiated for inoculation. Cytopathic changes were observed until the 11th passage (Fig.3A). At present, the virus GRF-1 has been passaged continuously to the 18th generation, with a virus titer of 104.5 TCID50/0.1 mL. Furthermore, the isolated GRF-1 porcine astrovirus in the cell culture medium was detected and genotyped by RT-PCR according to the previously described method. The results showed that the assay could detect porcine astrovirus (279 bp) in the cell culture medium during continuous passage of GRF-1 to more than 18 generations. Additionally, the genotype was identified as astrovirus type 4. Also, the PEDV and RV were not detected by RT-PCR. These results showed that the isolated PAstV-4-GRF-1 has been successfully purified, and its growth resulted in stable CPEs.

Electron microscopy showed that PAstV4-GRF-1 virions are nonenveloped and spherical, with a diameter of 30 nm. Additionally, star-like structures sur-

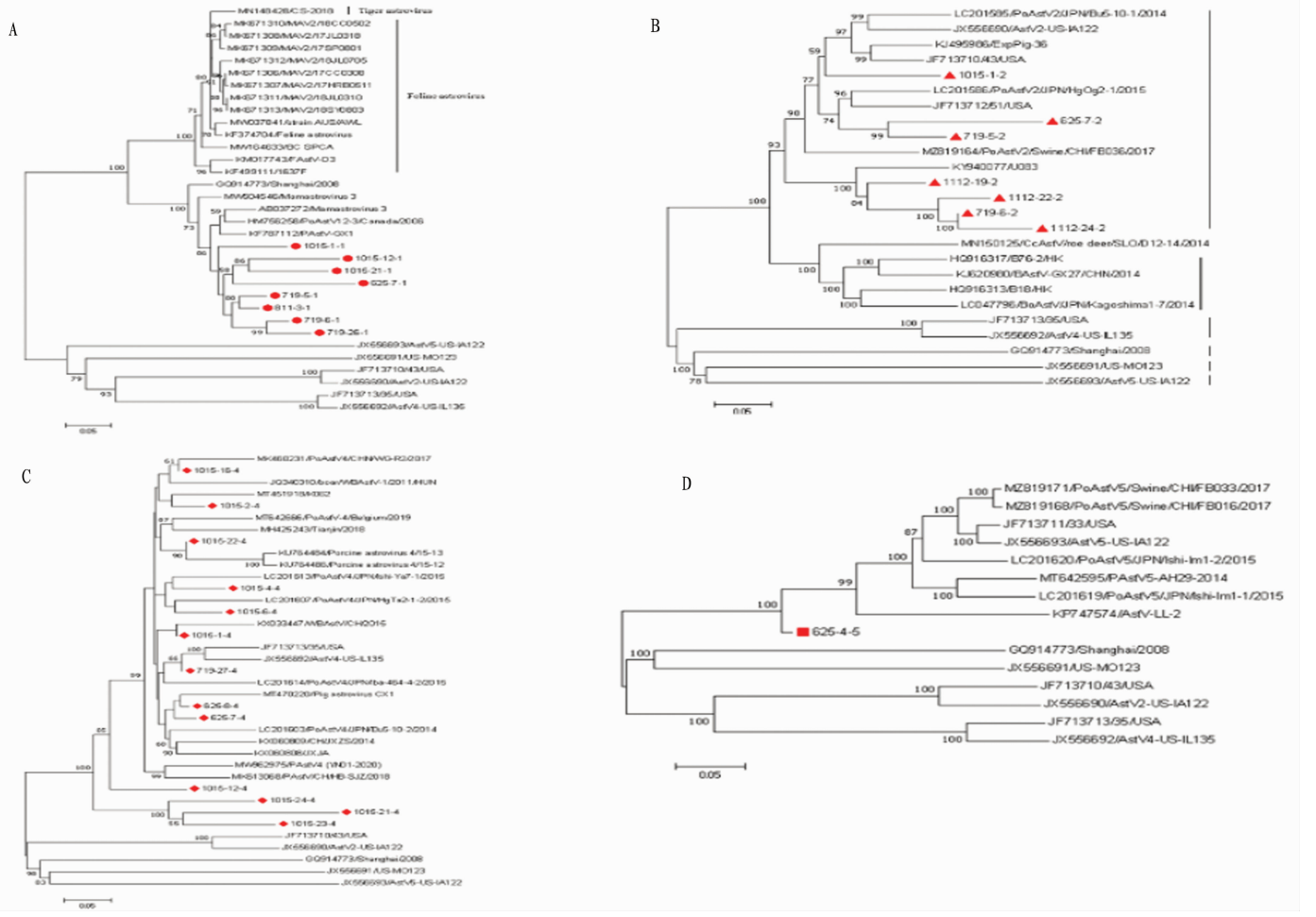

Fig.2 Phylogenetic analysis of the newly identified PAstV strains

Note: A. Phylogenetic analysis based on the identified PAstV-1 strain; B. Phylogenetic analysis based on the identified PAstV-2 strain; C. Phylogenetic analysis based on the identified PAstV-4 strain; D. Phylogenetic analysis based on the identified PAstV-5 strain. All strains were subjected to a comparative analysis with respect to AstV strains. A phylogenetic analysis was conducted on genome sequences of porcine astrovirus (PAstVs) and other representative mammal isolates. Homology analysis and tree construction were performed using the neighbor-joining method with the p-distance model in MEGA 7.0 software. The PAstV isolate analyzed in the present study is indicated by a circle in red. The GenBank accession number for each genome is provided.

rounding the icosahedral capsid were also observed, which is consistent with previous reports of human AstVs (Fig.3B).

2.4 Metaviral transcriptome sequencing In order to gain a deeper understanding of the evolutionary history of the recently identified PAstV strain, the samples of GRF-1 were subjected to macrovirus transcriptome sequencing. The RNA of the sample was sequenced using the Illumina second generation sequencing platform Novaseq 6000 (Fig.4A). Following the assembly procedure conducted with the Trinity splicing software, the resulting sequences were initially evaluated against the GenBank Virus RefSeq protein database using the DIAMOND software to identify any viral sequences. The screening results showed that a total of 37 333 610 Clean Reads were obtained, and 70 787 transcripts were assembled by means of the Trinity software. A total of 772 viral sequences were subjected to screening by comparing the spliced sequences with GenBank Virus RefSeq protein database using the DIAMOND software. A statistical analysis revealed that the proportion of Mamastrovirus in GRF samples was 88.30% based on species annotation, and PAstV-4 and PAstV-2 accounted for 21.79% and 0.32% by the species classification statistics (Fig.4B-C). Moreover, no other diarrhea-related viruses were identified in the sequencing results.

Finally, the obtained fragments were spliced, and the resulting ORF fragments were compared with the reference strains registered in GenBank. An evolutionary tree was constructed based on these comparisons. The results showed that the fragment c15230 was situated within the PAstV-4 ORF1a clade, and exhibited the highest degree of homology with the Tianjin strain MH425243, isolated in 2018 (Fig.4D). A genetic evolution analysis of ORF1b found that c15134 and c14913 were situated within the PAstV-2 clade, while c15397 was located within the PAstV-4 clade. Among the strains, c15134 exhibited the highest degree of homology with the Italian strain MG930777 in 2015, while

c14913 displayed the highest homology with the American strain JF713710 in 2010. The c15397 fragment exhibited the highest degree of homology with the Chinese Jiangsu strain MT470220, as determined in 2018, with a genetic distance of 0.259 (Fig.4E). For ORF2 fragments, c15357 was identified within the PAstV-2 clade, while c15176 and c15050 were located within the PAstV-4 clade. C15357 exhibited the shortest genetic distance from the 2010 American strains JF713712 and JX556690, with a genetic distance of 0.319. The genetic distance between c15176 and the 2014 Chinese Jiangxi strain KX060808 was 0.322, which was the shortest distance observed. The c15050 fragment was identical to the GRF-1 sequencing fragment of the isolated strain and exhibited the highest degree of homology with the Hunan PAstV-4 sequence MK460231 in China. The strain GRF-1, isolated from PK-15 cells, was further verified to be PAstV-4 (Fig.4F).

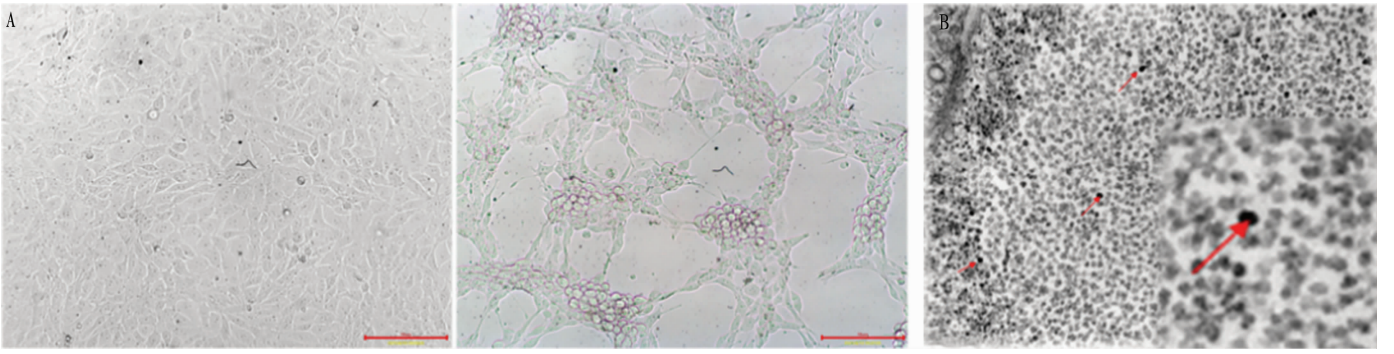

Fig.3 The pathogenicity of PAstV-4-GRF-1 to PK-15 cells

Note: A. The cells infected with the 11th passage of PAstV-4 GRF-1 exhibited a larger and rounder morphology, with gradual breakage and detachment (right). The control without virus is shown in the left image (bar=10 μm); B. Electron microscopy showed that GRF-1 virions are non-enveloped and spherical, with a diameter of 30 nm (×60 000).

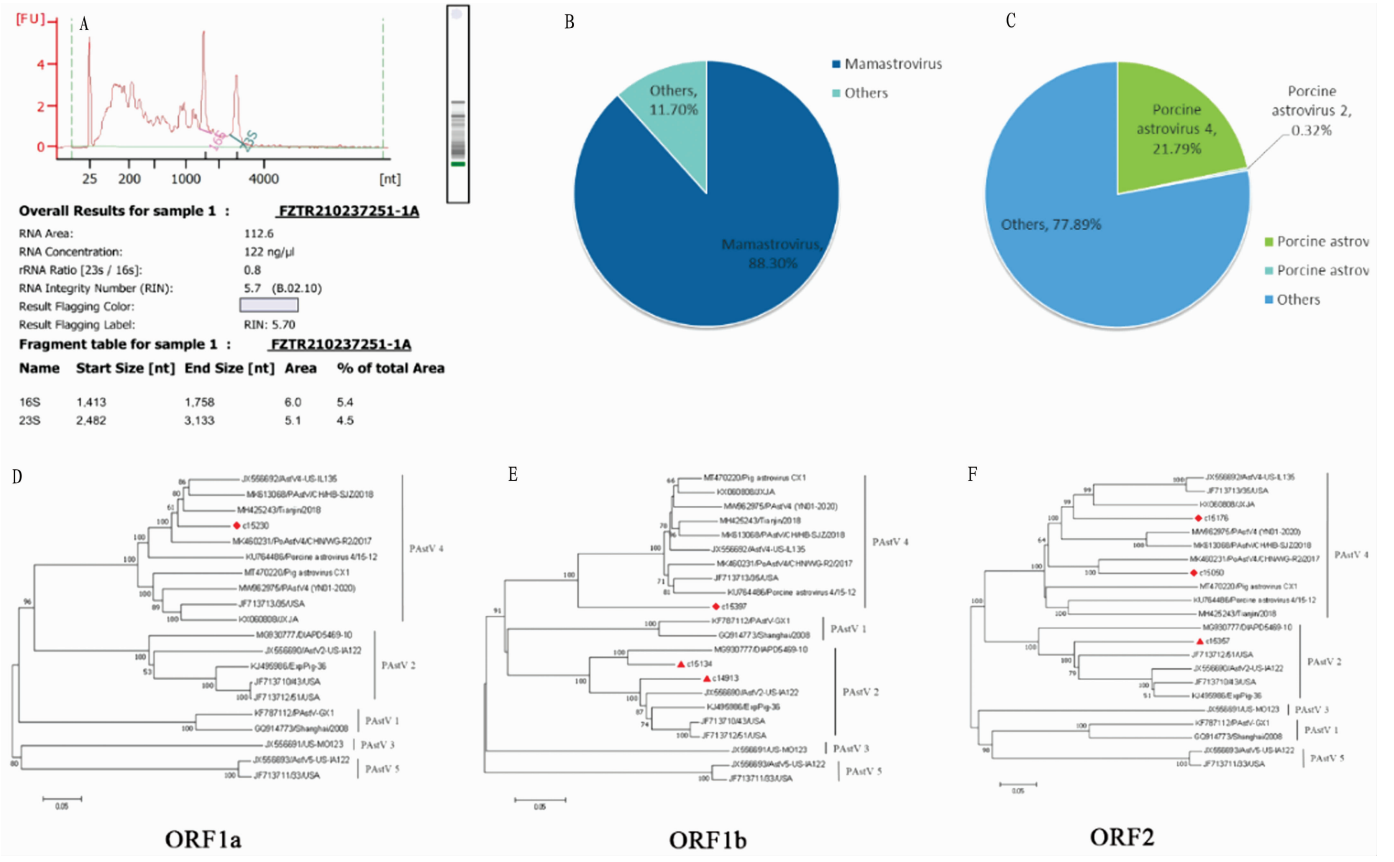

Fig.4 Metaviral transcriptome sequencing of isolated strain GRF-1

Note: A. RNA quality control results; B. Genus classification statistics of GRF-1; C. Species classification statistics of PAstV; D. Phylogenetic tree based on the ORF 1a genes with the reference strains; E. Phylogenetic tree based on the ORF 1b genes with the reference strains; F. Phylogenetic tree based on the ORF2 genes with the reference strains. The phylogenetic tree was constructed using the neighbor-joining method with the p-distance substitution model, 1 000 bootstrap replicates and a 70% cut-off value. The sequences of GRF-1 are indicated by the red circle. The software utilized was MEGA 7.0.

3 Discussion

Astroviruses are non-enveloped, positive single-stranded RNA viruses that have the potential to infect a wide range of mammals, including humans, as well as avian species such as ducks. Astroviruses exhibit a high degree of host range and are prone to rapid genetic variations, which enhance their capacity to spread across species and adapt to new hosts. It has been identified in 31 species of mammals and 6 species of poultry, with the host range continuing to expand. Porcine astroviruses (PAstVs) belong to the genus of mammalian astrovirus, which has a global distribution. In recent years, the prevalence of PAstV in Chinese pig farms has increased. However, there is a paucity of literature on the role of porcine astrovirus in Shandong Province, China.

The objective of this study was to investigate the prevalence of porcine astrovirus in porcine diarrhea cases and healthy pigs in Shandong province. RT-PCR results showed that the total positive rate of PAstV in porcine diarrhea cases collected from certain regions of Shandong province from January 2021 to October 2023 was 34.6% (355/1025), while the total positive rate of PAstV in healthy pigs was 8.13% (69/849). Of the five known PAstV lineages, PAstV-3 is primarily associated with neurological symptoms, while other types are linked to diarrhea. It has been reported that PAstV-2 and PAstV-4 are the predominant genotypes reported in Canada, Hungary, China, USA, South Korea, Croatia, Italy, Kenya, Austria, Germany, Spain, and Sweden. In recent years, PAstV-5 has become increasingly prevalent. The results of this study are consistent with previous findings, which have identified five distinct PAstV lineages in cases of diarrhea. Notably, no PAstV-3 strains were detected in the samples analyzed in this study. The proportion of PastV-1, PastV-2, PastV-4, and PastV-5 was found to be 25.4%, 28.2%, 35.2%, and 22.5%, respectively. The proportions of PAstV-1, PAstV-2 and PAstV-4 in healthy pig samples were 27.5%, 37.7%, and 40.6%, respectively. It seems that PAstV-4 exhibited the highest infection rate and was the dominant strain in Shandong province. Furthermore, it was observed that two or more distinct genotypes were present in mixed infections, suggesting the potential for genetic recombination between different genotypes of PAstV. As RNA viruses, there have been numerous reports of genetic variety and recombination events in human AstVs. However, few recombination events have been identified within PAstV genotypes. Furthermore, past studies have indicated that PAstV can co-infect with other enteroviruses, which has been associated with a reduction in the survival rate of piglets. This has the potential to result in significant economic losses for the pig industry. We found that in PAstV-positive samples, the co-infection rates of PEDV and PRV in diarrhea-infected piglets were 46.5% and 42.3%, respectively, while the co-infection rates of PEDV and PRV in healthy piglets were 47.8% and 43.5%, respectively. These findings suggest that the co-infection of PEDV and PRV may play a potential role in the pathogenesis of PEDV-induced diarrhea in piglets.

A phylogenetic analysis of 29 sequenced samples revealed a significant genetic distance within the intra-genotypic populations of PAstV-1, PAstV-2 and PAstV-4. In particular, PAstV-4 is found in 8 different branches of the evolutionary tree of this genotype, which suggests that there may be a significant genetic variation of this strain in different regions of Shandong Province. In addition, it was discovered that there were significant genetic variations between the PAstV-4 strains isolated in this study and those from different countries. This phenomenon was also observed in PAstV-1 and PAstV-2, which may be attributed to the introduction of breeding pigs from abroad. Moreover, this study found that PAstV-1 is evolutionarily related to feline astrovirus (FeAstV) and tiger astrovirus (TigAstV) in the phylogenetic tree, while PAstV-2 is related to *Capreolus capreolus* astrovirus (CcAstV) and bovine astrovirus (BoAstV). This suggests that PAstV may have undergone cross-species transmission.

The pathogenicity of PAstVs has been the subject of only a limited number of studies due to the difficulty of isolating the virus in cells. To date, only a few strains have been successfully isolated using cell lines, including a porcine astrovirus strain on PK-15 cells, which was isolated by Xiaogui Shang in 2010. This strain was found to cause cytopathic changes. In 2014, Liu Huan successfully isolated the PAstV-GX1 virus from pig feces in Guangxi. In 2020, a swine astrovirus type 5 was isolated from clinical swine fever virus (CSFV)-infected tissue samples. This isolation may have been influenced by the CSFV counter infection, which significantly enhanced the replication of PAstV-5[21]. To date, the most prevalent strains of porcine astrovirus (PAstV-2 and PAstV-4) in China have not been isolated. In this experiment, 30 samples with a higher viral load were selected from the 424 samples with a positive detection of porcine astrovirus for virus isolation. By investigating the concentration of trypsin and optimizing the culture conditions on PK-15 cells, a porcine astrovirus type 4 strain, designated GRF-1, was successfully isolated through the use of pathological changes, RT-PCR and sequencing. Following the infection of PK-15 cells for a period of 24 h, the cells exhibited morphological changes, becoming large, round, and fragmented. Thereafter, the cell culture medium was subjected to three cycles of freezing and thawing, after which RNA was extracted. The target band of porcine astrovirus was successfully detected by RT-PCR, whereas the blank control cells of astrovirus could not be detected. At the same time, other diarrheal diseases (PEDV and RV) were de-

tected in the cell culture medium and found to be negative, thus excluding the possibility that the observed cytopathic changes were caused by PEDV and RV. The phylogenetic tree showed the strain exhibited the highest degree of homology (95.4%) with the Chinese Hunan strain MK460231, as documented in 2017.

Unfortunately, the lack of monoclonal antibodies limits our ability to further investigate the pathogenicity of PAstV-4-GRF1. Consequently, the clinical information will be obtained from the isolated strains. However, the isolation of this strain has provided a crucial material for further studies into the role of PAstV-4 in diseases of pigs.

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